

Protective effect of carvacrol on liver injury in type 2 diabetic db/db mice

WEI ZHAO^{1*}, LI CHEN^{2*}, HENG ZHOU^{1*}, CHUNYAN DENG¹, QIZHEN HAN¹,
YONGHUA CHEN¹, QING WU¹ and SHANSHAN LI¹

Departments of ¹Endocrinology and ²Prevention and Health Care, The Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, Guiyang, Guizhou 550003, P.R. China

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Abstract. The present study aimed to investigate the protective effect of carvacrol on liver injury in mice with type 2 diabetes mellitus (T2DM) and to assess its potential molecular mechanism. Mice were divided into three groups (n=15/group): Non-diabetic db/m+ mice group, db/db mice group and db/db mice + carvacrol group. In the db/db mice + carvacrol group, db/db mice were administered 10 mg/kg carvacrol daily by gavage for 6 weeks. Fasting blood glucose and insulin levels were separately examined. Pathological changes were observed using hematoxylin and eosin, Masson's trichrome, periodic acid Schiff and reticular fiber staining. In addition, immunohistochemistry, immunofluorescence and western blotting were used to examine the expression levels of Toll-like receptor 4 (TLR4), NF- κ B, NALP3, AKT1, phosphorylated (p)-AKT1, insulin receptor (INSR), p-INSR, mTOR, p-mTOR, insulin receptor substrate 1 (IRS1) and p-IRS1 in the liver tissues. The results revealed that carvacrol improved blood glucose and insulin resistance of T2DM db/db mice. After treatment with carvacrol for 6 weeks, the serum levels of TC, TG and LDL-C were markedly reduced, whereas HDL-C levels were significantly increased in db/db mice. Furthermore,

carvacrol administration significantly decreased serum ALT and AST levels in db/db mice. Serum BUN, Cre and UA levels were markedly higher in db/db mice compared with those in the control group; however, carvacrol treatment markedly reduced their serum levels in db/db mice. Furthermore, histological examinations confirmed that carvacrol could protect the liver of db/db mice. Carvacrol could ameliorate liver injury induced by T2DM via mediating insulin, TLR4/NF- κ B and AKT1/mTOR signaling pathways. The present findings suggested that carvacrol exerted protective effects on the liver in T2DM db/db mice, which could be related to insulin, TLR4/NF- κ B and AKT1/mTOR signaling pathways.

Introduction

Type 2 diabetes mellitus (T2DM) is a common chronic metabolic disease (1). It is estimated that there are ~382 million patients with T2DM worldwide. By 2035, it is estimated that the number of patients will be 592 million (2). The liver is one of the target organs affected by long-term hyperglycemia (3). Pronounced insulin resistance and hyperglycemia have been observed in patients with T2DM accompanied by nonalcoholic fatty liver disease (NAFLD) (4). Lipid accumulation caused by insulin resistance can promote the development of NAFLD in patients with T2DM (5-7). Furthermore, inflammation and oxidative stress caused by liver lipid accumulation can accelerate liver cell necrosis and fibrosis (8,9). Currently, the main treatment options for T2DM or NAFLD include exercise, diet, and hypoglycemic and hypolipidemic treatment; however, the therapeutic effects of these strategies remain unsatisfactory (10). Increasing evidence has suggested that the occurrence and development of T2DM-induced NAFLD is associated with a complex pathogenesis and multiple factors (10). There is currently no effective drugs for the treatment of T2DM-induced NAFLD; therefore, there is an urgent need to identify novel drugs to prevent NAFLD caused by T2DM.

Through in-depth research, it has been revealed that numerous active ingredients in traditional Chinese medicine have the potential to relieve NAFLD. Increasing evidence has demonstrated that carvacrol (2-methyl-5-isopropylphenol), a monoterpenic phenol, may possess pharmacological properties, including neuroprotective (11), anti-inflammatory (12),

Correspondence to: Professor Wei Zhao, Department of Endocrinology, The Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, 32 Feishan Street, Yunyan, Guiyang, Guizhou 550003, P.R. China
E-mail: doctorzhaowei@126.com

*Contributed equally

Abbreviations: T2DM, type 2 diabetes; NAFLD, nonalcoholic fatty liver disease; OGTT, oral glucose tolerance test; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglyceride; GSP, glycosylated serum protein; BUN, blood urea nitrogen; Cre, creatinine; UA, uric acid; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff

Key words: carvacrol, liver injury, T2DM, blood glucose, insulin resistance

anti-oxidative (13), anti-bacterial (14) and anti-cancer (15) effects. Carvacrol has been reported to relieve T2DM-related complications, such as vascular inflammation (12), hypercontractility in the aorta (16) and diabetic cardiomyopathy (17). Nevertheless, to the best of our knowledge, the effect of carvacrol on T2DM-induced liver injury remains unknown. Because hyperglycemic db/db mice have been widely utilized as T2DM models, the present study chose this method to construct T2DM models (18,19). The present study hypothesized that carvacrol may ameliorate liver injury induced by T2DM.

Materials and methods

Animals. A total of 30 male C57BL/KsJ db/db mice (age, 8 weeks; weight, 32–36 g) and 15 age-matched C57BL/6J control non-diabetic db/m+ mice (weight, 16–18 g) were purchased from Changzhou Cavans Experimental Animal Co., Ltd. (license number: SCXK2001-0003). The mice were maintained under a 12-h light/dark cycle, at 22±3°C and 70±10% humidity; they could freely move and had free access to food and water. The animal experiment was conducted according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (20). The present study was approved by the Animal Care and Ethics Committee of The Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine (approval no. 2018072; Guiyang, China).

Grouping and administration. All mice were routinely fed for 3 days. The 15 db/m+ mice were employed as the normal control group, and db/db mice were randomly separated into two groups (n=15/group): Model group and carvacrol group. Mice in the model and normal control groups were administered saline daily by gavage. Mice in the carvacrol group were administered an appropriate dose of carvacrol (10 mg/kg) daily by gavage, as previously described (12). After daily treatment for 6 weeks, all mice were fasted for 6 h, but were allowed to drink water. Subsequently, venous blood was obtained from the tail vein and fasting blood glucose was determined using an automatic blood glucose meter (Johnson & Johnson). After taking blood, the mice were euthanized through intraperitoneal injection of 200 mg/kg pentobarbital sodium. Absence of a heartbeat and breathing was used as the criterion for confirming euthanasia of rats. The blood samples were left at room temperature for 2 h, and centrifuged at 3,500 x g for 15 min at 4°C. The supernatant was harvested for serological analysis. In addition, liver tissues were removed and weighed. Liver index was calculated in line with the ratio of liver weight (g) and body weight (g). The tissue specimens were stored at -80°C before further use.

Determination of serum glucose and insulin. Mouse serum insulin concentration was examined using a mouse insulin ELISA kit (cat. no. H203-1-2; Nanjing Jiancheng Bioengineering Institute) according to manufacturer's protocol. The absorbance value was examined at 450 nm using a microplate reader. Homeostasis model assessment of insulin resistance (HOMA-IR) is one of the most common methods used to evaluate insulin resistance (21). HOMA-IR was determined according to the following equation:

$HOMA-IR = \text{fasting blood glucose (mmol/l)} \times \text{fasting insulin (mU/l)} / 22.5$, where fasting insulin was detected using the mouse insulin ELISA kit.

Oral glucose tolerance test (OGTT). After fasting for 6 h, the mice were given an oral glucose solution (1.2 g/kg; body weight). Blood was drawn from the tail vein and blood glucose was determined after glucose administration for 0, 30, 60 and 120 min using a glucose monitor (Ascensia ELITE; Bayer).

Insulin tolerance test (ITT). ITT was conducted via intraperitoneally injecting 1 U/kg regular insulin (Novo Nordisk A/S) into mice that were fasted for 6 h. Blood glucose levels were determined at 0, 15, 30, 60 and 120 min using a glucose monitor (Ascensia ELITE; Bayer).

Biochemical analyses. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), glycosylated serum protein (GSP), blood urea nitrogen (BUN), creatinine (Cre) and uric acid (UA) levels were separately detected using ALT (cat. no. C009-2-1), AST (cat. no. C010-1-1), TC (cat. no. A111-1-1), LDL-C (cat. no. A113-1-1), HDL-C (cat. no. A112-1-1), TG (cat. no. A110-1-1), GSP (cat. no. A037-2-1), BUN (cat. no. C013-2-1), Cre (cat. no. C011-2-1) and UA ELISA kits (cat. no. C012-2-1) (all from Nanjing Jiancheng Bioengineering Institute) according to manufacturer's protocols.

Hematoxylin and eosin (H&E) staining. Liver tissue specimens were fixed in 4% paraformaldehyde solution at 4°C for 24 h, embedded in paraffin and sectioned. Paraffin-embedded sections (5 μm) were treated with xylene for 20 min twice, absolute ethanol for 5 min twice and 75% ethanol for 5 min at 37°C. The sections were separately stained with 0.5% hematoxylin and 0.5% eosin solution for 5 min at 37°C. Neutral gum was used for sealing and images were captured under an optical microscope (Olympus Corporation). The nucleus was stained blue and the cytoplasm was stained red.

Masson's trichrome staining. Liver tissues were fixed overnight with 10% formalin solution at 4°C and cut into 5-μm sections. Masson's trichrome staining was conducted using the Masson's Trichrome Staining Kit (cat. no. M1340; Beijing Solarbio Science & Technology Co., Ltd.). The tissues were stained with 0.5% Regaud hematoxylin for 10 min at 37°C. After washing for 15 min, 2% Masson lichun red acid complex red fluid was added for 5–10 min. Subsequently, the tissues were stained with 0.5% aniline blue, and then immersed in 1% phosphotungstic acid-phosphomolybdic acid solution for 15 min. The tissues were then washed with 1% glacial acetic acid solution for 5 min, and were further washed with 1% glacial acetic acid solution, dehydrated, permeabilized and sealed in neutral resin. Images were captured under a light microscope (Olympus Corporation). The nuclei were stained black and the collagen fibers were stained blue.

Reticular fiber staining. Liver tissues were fixed overnight with 10% formalin solution at 4°C and cut into 5-μm sections. Liver

sections were incubated with 0.5% potassium permanganate for 3 min, and 2% oxalic acid and 2% ferric ammonium sulfate for 1 min at 37°C. Subsequently, the sections were treated with ammonia silver solution for 5 min and 10% formaldehyde solution for 30 sec. The sections were then incubated with the gold chloride solution for 5 min, and sodium thiosulfate for 2 min. Finally, the sections were stained with 0.5% eosin staining solution for 30 sec, and images were captured under a light microscope (Olympus Corporation) and were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Periodic acid Schiff (PAS) staining. Liver tissues were fixed, embedded and sectioned as they were prior to H&E staining. Paraffin-embedded sections were stained with 1% periodic acid for 15 min, 1% Chevron dye for 30 min and 0.5% hematoxylin for 3-5 min at 37°C. Images were captured under a light microscope (Olympus Corporation). The glycogen was stained purple-red and the cell nuclei were stained light blue.

Immunohistochemistry. Liver tissues were fixed overnight with 10% formalin solution at 4°C and cut into 5- μ m sections. Liver tissue sections were blocked with 5% skimmed milk powder for 1 h at 37°C. Subsequently, the sections were incubated with the following primary antibodies: Toll-like receptor 4 (TLR4; 1:100; cat. no. ab13556; Abcam), NF- κ B (1:100; cat. no. 10745-1-AP; Proteintech Group, Inc.), NALP3 (1:100; cat. no. bs-6655R; BIOSS), AKT1 (1:150; cat. no. 10176-2-AP; Proteintech Group, Inc.), phosphorylated (p)-AKT1 (1:100; cat. no. 66444-1-Ig; Proteintech Group, Inc.), insulin receptor (INSR; 1:80; cat. no. ab69508; Abcam), p-INSR (1:80; cat. no. bs-16680R; BIOSS), mTOR (1:100; cat. no. 20657-1-AP; Proteintech Group, Inc.), p-mTOR (1:200; cat. no. ab109268; Abcam), insulin receptor substrate 1 (IRS1; 1:100; cat. no. ab245314; Abcam) and p-IRS1 (1:100; cat. no. bs-3200R; BIOSS) at 4°C overnight. Subsequently, sections were incubated with secondary antibodies (1:500; cat. nos. ab205719 and ab6721; Abcam) for 1 h at 37°C, followed by staining with DAB for 5 min and hematoxylin for 2 min at 37°C. Images were captured under a light microscope (Olympus Corporation).

Immunofluorescence. Liver tissues were fixed overnight with 10% formalin solution at 4°C and cut into 5 μ m sections. Liver tissue sections were blocked with 5% skimmed milk powder for 1 h at 37°C treated with unconjugated primary antibodies, as follows: NALP3 (1:100; cat. no. bs-6655R; BIOSS), mTOR (1:100; cat. no. 20657-1-AP; Proteintech Group, Inc.), p-mTOR (1:200; cat. no. ab109268; Abcam), TLR4 (1:100; cat. no. ab13556; Abcam), AKT1 (1:150; cat. no. 10176-2-AP; Proteintech Group, Inc.) and p-AKT1 (1:100; cat. no. 66444-1-Ig; Proteintech Group, Inc.) overnight at 4°C. After washing with TBS-0.3% Tween-20 (TBST), sections were incubated with secondary antibodies (1:500; cat. nos. ab150077 and ab150113; Abcam) at room temperature for 2 h. Images were captured under an immunofluorescence microscope.

Western blotting. Tissues were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) plus phosphatase and protease inhibitors on ice. After centrifugation at 12,000 x g

for 15 min at 4°C, the supernatant was harvested. Protein concentration was examined using the BCA method (Pierce; Thermo Fisher Scientific, Inc.). Subsequently, 120 μ g protein was loaded per lane, samples were separated by SDS-PAGE on 10% gels and were transferred onto PVDF membranes (cat. no. IPVH00010; EMD Millipore). The membranes were blocked with 5% skimmed milk powder for 2-3 h at room temperature. The membranes were then incubated with the following primary antibodies: TLR4 (1:1,000; cat. no. ab13556; Abcam), NF- κ B (1:1,000; cat. no. 10745-1-AP; Proteintech Group, Inc.), NALP3 (1:500; cat. no. bs-6655R; BIOSS), AKT1 (1:1,000; cat. no. 10176-2-AP; Proteintech Group, Inc.), p-AKT1 (1:500; cat. no. 66444-1-Ig; Proteintech Group, Inc.), INSR (1:400; cat. no. ab69508; Abcam), p-INSR (1:400; cat. no. bs-16680R; BIOSS), mTOR (1:2,000; cat. no. 20657-1-AP; Proteintech Group, Inc.), p-mTOR (1:1,000; cat. no. ab109268; Abcam), IRS1 (1:500; cat. no. ab245314; Abcam), p-IRS1 (1:500; cat. no. bs-3200R; BIOSS) and β -actin (1:1,000; cat. no. bs-0061R; BIOSS) at 4°C overnight. After washing three times with TBST, the membranes were incubated with secondary antibodies (1:2,000; cat. nos. ab205719 and ab6721; Abcam) at room temperature for 1 h. Blots were visualized utilizing an ECL kit (cat. no. KGP1121; Nanjing KeyGen Biotech Co., Ltd.) and a gel image detection analyzer (Invitrogen; Thermo Fisher Scientific, Inc.). The results were semi-quantified using ImageJ software (version 1.48; National Institutes of Health).

Statistical analysis. GraphPad Prism 8.0 (GraphPad Software, Inc.) was utilized for statistical analysis. Data are presented as the mean \pm standard deviation and each assay was repeated at least three times. One-way ANOVA was used for multiple comparisons, followed by Tukey's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Carvacrol decreases body weight, liver weight and liver index of db/db mice. Compared with in the control group, the body weight of db/db mice was significantly increased (Fig. 1A). Following treatment with 10 mg/kg carvacrol, the body weight of db/db mice was gradually decreased. Compared with that in the control group, db/db mice had a significantly higher daily intake of water (Fig. 1B) and feed (Fig. 1C). Notably, carvacrol treatment could ameliorate daily water intake for db/db mice; however, there was no significant difference in feed intake between the model and carvacrol groups. Furthermore, liver weight and liver index of db/db mice were significantly increased compared with those in the control group (Fig. 1D and E). After 6 weeks of treatment with 10 mg/kg carvacrol, these two indexes were significantly decreased in db/db mice (Fig. 1D and E). These results revealed that T2DM could increase body and liver weight, which could be reversed by carvacrol treatment.

Carvacrol improves blood glucose and insulin resistance of T2DM db/db mice. Fasting blood glucose and insulin levels can reflect sensitivity to insulin at an overall level (22). Compared with in the control group, the db/db mice exhibited higher fasting blood glucose levels (Fig. 2A). Conversely, after

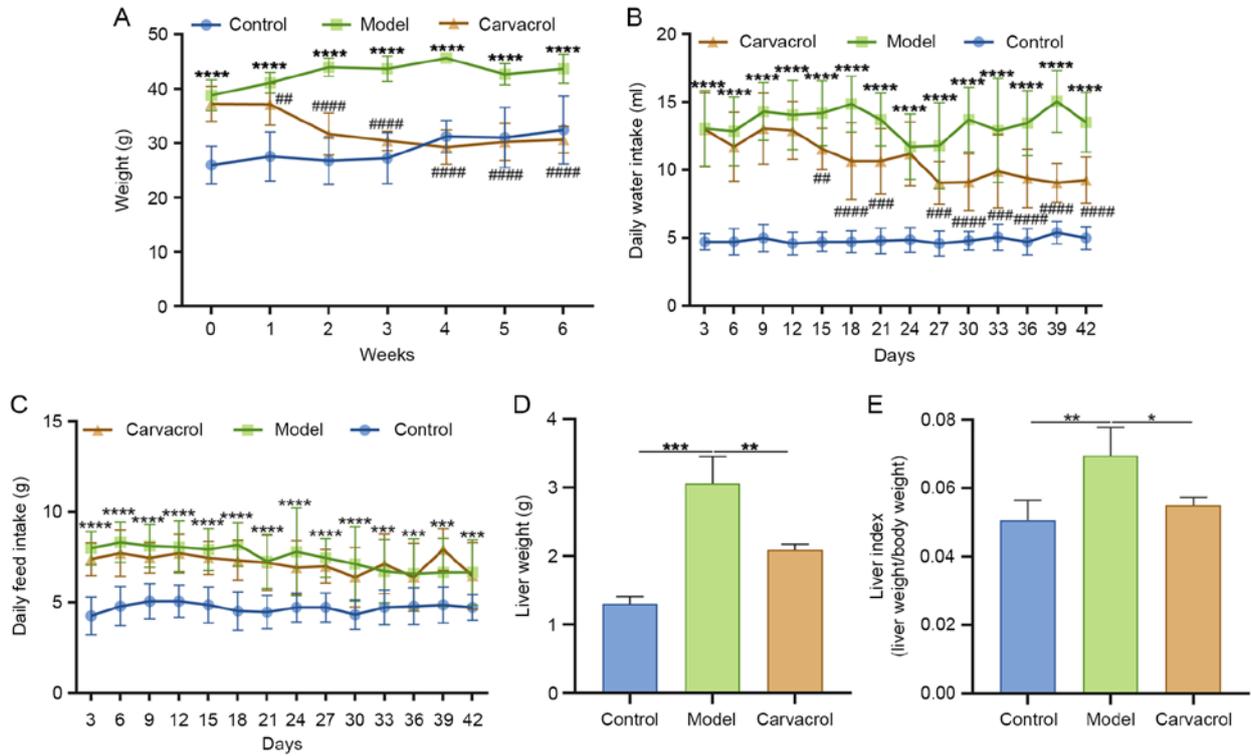


Figure 1. Effect of carvacrol on body weight, energy intake, liver weight and liver index of db/db mice. (A) Body weight, (B) daily water intake, (C) daily feed intake, (D) liver weight and (E) liver index. $n=15/\text{group}$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ vs. control group or as indicated; ## $P<0.01$, ### $P<0.001$, #### $P<0.0001$ vs. model group.

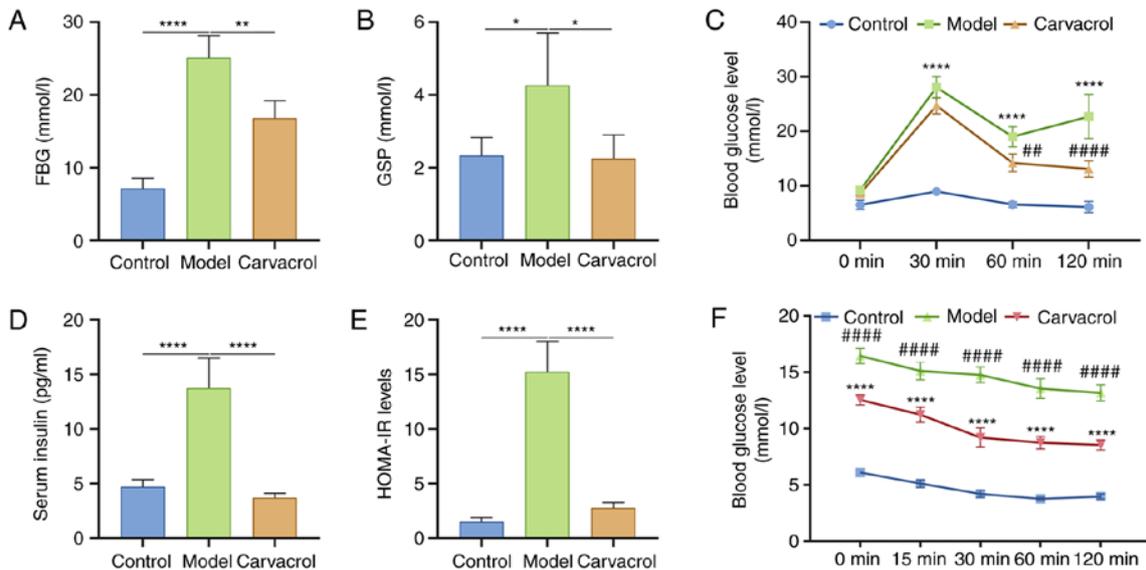


Figure 2. Carvacrol improves FBG and insulin resistance in db/db mice. (A) FBG, (B) GSP, (C) blood glucose level, as determined by OGTT, (D) fasting insulin, (E) HOMA-IR and (F) blood glucose level, as determined by ITT. $n=15/\text{group}$. * $P<0.05$, ** $P<0.01$, **** $P<0.0001$ vs. control group or as indicated; ## $P<0.01$, ### $P<0.0001$ vs. model group. FBG, fasting blood glucose; GSP, glycosylated serum protein; HOMA-IR, homeostasis model assessment of insulin resistance; ITT, insulin tolerance test; OGTT, oral glucose tolerance test.

6 weeks of administration, carvacrol significantly reduced fasting blood glucose levels (Fig. 2A). Furthermore, GSP levels were detected in the three groups. As shown in Fig. 2B, the db/db mice had higher GSP levels compared with those in the control group. Carvacrol administration significantly decreased the levels of GSP in db/db mice. In addition, an OGTT was performed on the mice; as expected, carvacrol

markedly decreased the blood glucose levels of db/db mice (Fig. 2C). Furthermore, fasting blood insulin levels were significantly higher in the model group compared with those in the control group (Fig. 2D), which were decreased by carvacrol treatment. HOMA-IR levels were also significantly increased in the model group compared with those in the control group (Fig. 2E). Conversely, HOMA-IR levels were

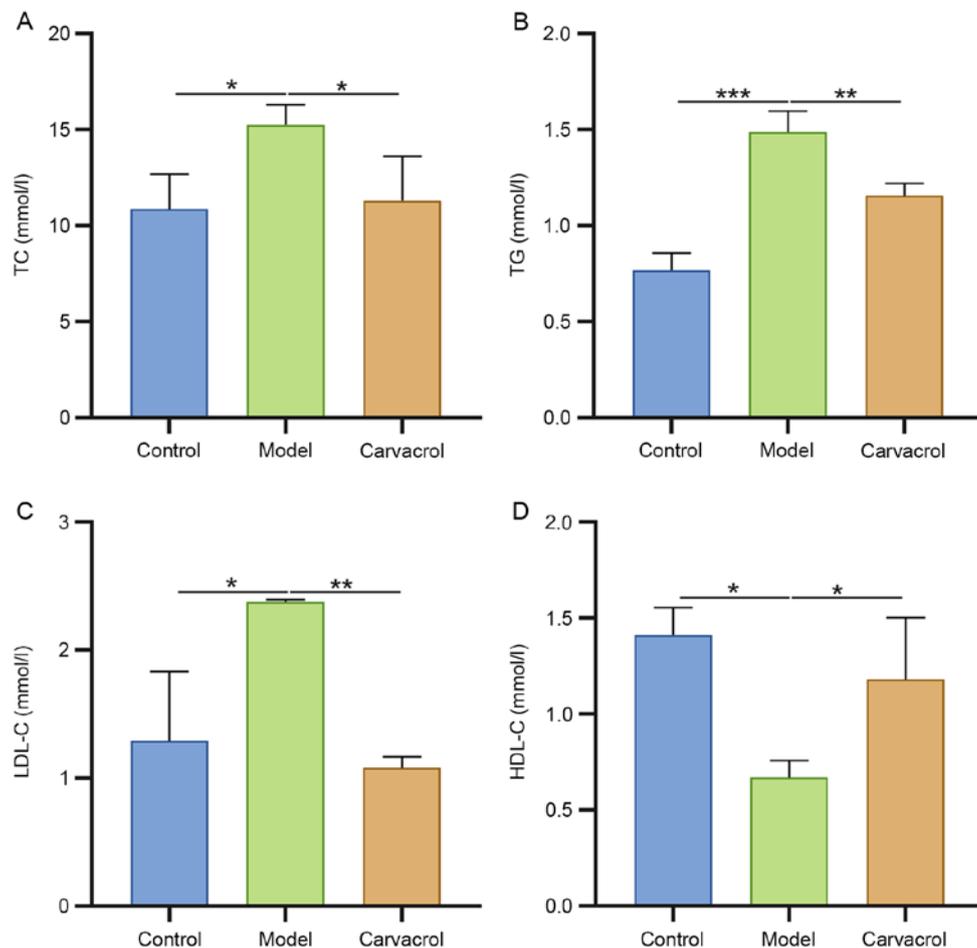


Figure 3. Effects of carvacrol on the serum levels of (A) TC, (B) TG, (C) LDL-C and (D) HDL-C for db/db mice. n=15/group. *P<0.05, **P<0.01, ***P<0.001. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride.

significantly suppressed in db/db mice following carvacrol treatment. The mice also underwent an ITT. As shown Fig. 2F, compared with in the model group, blood glucose levels were significantly decreased in carvacrol-treated db/db mice at 0, 15, 30, 60 and 120 min after insulin injection.

Carvacrol ameliorates dyslipidemia in db/db mice. TC (Fig. 3A), TG (Fig. 3B) and LDL-C levels (Fig. 3C) were significantly increased, and HDL-C levels (Fig. 3D) were significantly decreased in the serum of db/db mice compared with those in the control group. Carvacrol administration significantly reduced serum levels of TC, TG and LDL-C, and increased serum levels of HDL-C in db/db mice, indicating that carvacrol could ameliorate hyperlipidemia of db/db mice.

Carvacrol improves liver and kidney damage in db/db mice. When liver cells are damaged, a large amount of AST and ALT is released from the liver cells into the blood (23). Therefore, serum AST and ALT levels can reflect the severity of liver damage. As shown in Fig. 4A and B, serum ALT and AST levels were significantly higher in db/db mice compared with those in the control group. Following administration with carvacrol, serum ALT and AST levels were significantly reduced in db/db mice, suggesting that carvacrol could improve T2DM-induced liver injury. Furthermore, the renal function of the three groups was assessed. The results revealed that serum

BUN (Fig. 4C), Cre (Fig. 4D) and UA levels (Fig. 4E) were significantly higher in db/db mice compared with those in the control group. Conversely, carvacrol treatment significantly lowered these serum levels in db/db mice. These findings demonstrated that carvacrol could improve liver and kidney damage in db/db mice.

Carvacrol protects the liver of db/db mice, as determined through histological examinations. Histological analysis of liver cell injury was performed using H&E staining of the three groups (Fig. 5A). It was revealed that liver injury of db/db mice was more serious compared with that in the control group. Notably, vacuolar degeneration of hepatocytes was detected in db/db mice. Conversely, carvacrol reduced the levels of liver cell damage in db/db mice. Collagen fiber content in liver tissues was detected utilizing Masson's trichrome staining (Fig. 5B). It was demonstrated that control mice exhibited a normal liver lobule structure, whereas obvious hyperplasia of collagen fibers in liver tissue was detected in db/db mice. Following carvacrol treatment, the amount of collagen fiber hyperplasia in the liver tissue of db/db mice was markedly reduced. These findings indicated that diabetes may cause liver fibrosis and carvacrol treatment may improve collagen fiber hyperplasia in the liver tissues of db/db mice. In addition, PAS was performed to detect glycogen in liver cells (Fig. 5C). Compared with in the

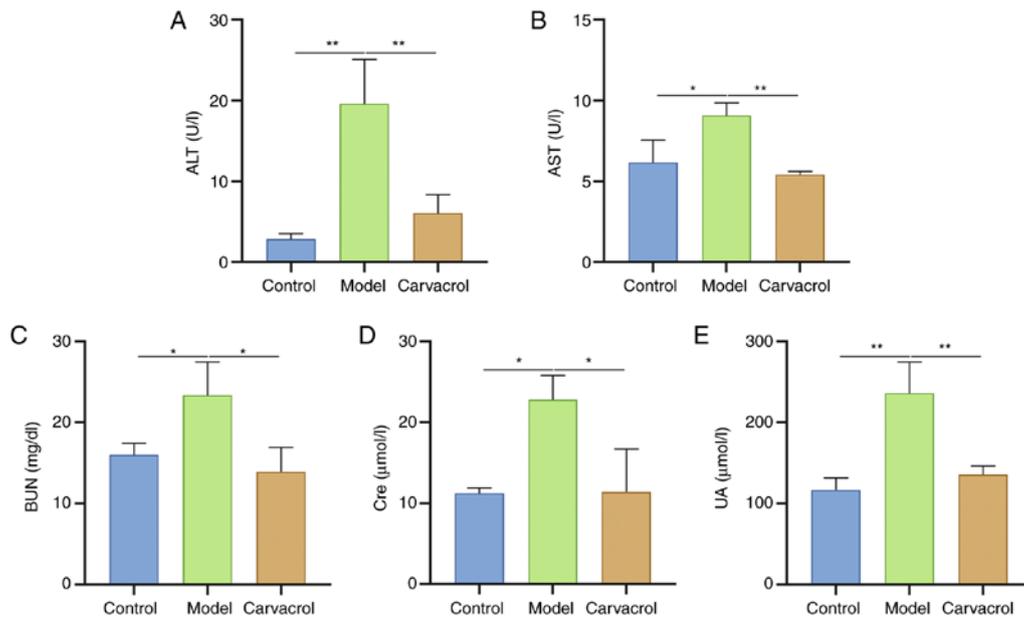


Figure 4. Carvacrol improves liver and kidney damage in db/db mice. (A) Serum ALT, (B) AST, (C) BUN, (D) Cre and (E) UA levels were determined in the control, model and carvacrol groups. $n=15/\text{group}$. * $P<0.05$, ** $P<0.01$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Cre, creatinine; UA, uric acid.

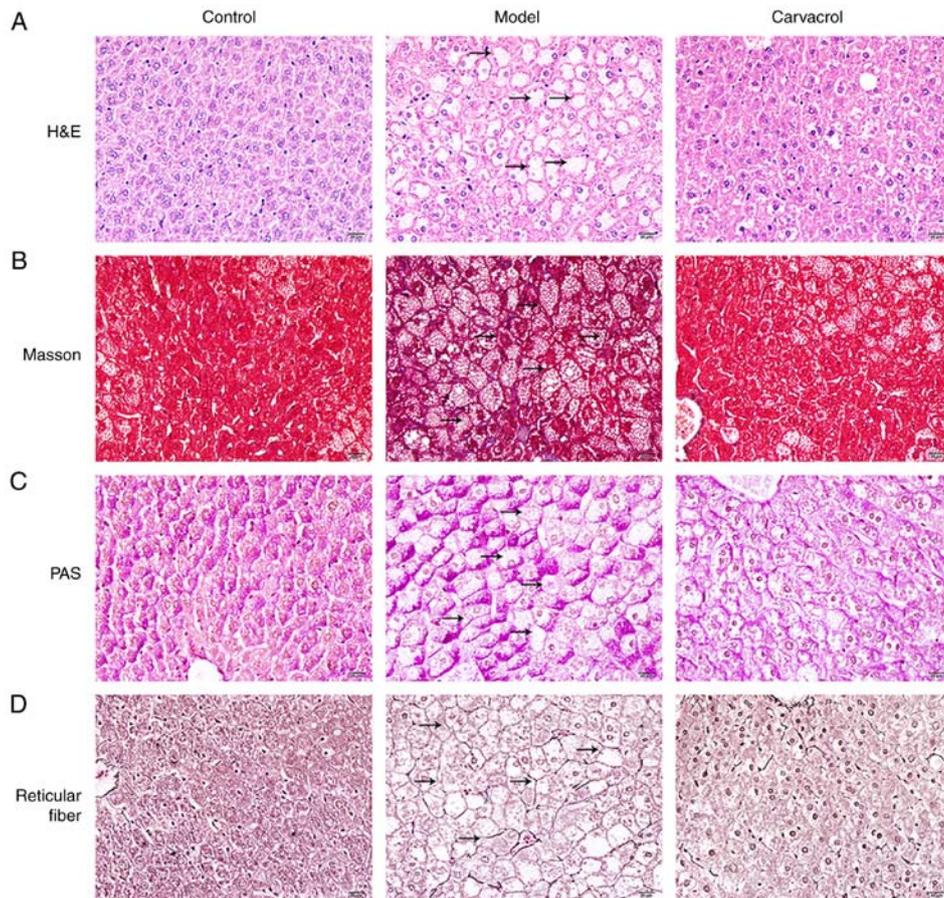


Figure 5. Carvacrol protects the liver of db/db mice through histological examinations. (A) H&E staining. (B) Masson's trichrome staining. (C) PAS. (D) Reticular fiber staining. Scale bar, $20\ \mu\text{m}$; magnification, $\times 200$. $n=15/\text{group}$. Arrows indicate (A) liver cell damage, (B) collagen fiber hyperplasia, (C) glycogen accumulation and (D) reticular fiber hyperplasia. H&E, hematoxylin and eosin; PAS, periodic acid Schiff.

control group, there was obvious glycogen accumulation in the cytoplasm of liver cells in db/db mice, whereas carvacrol

treatment markedly ameliorated glycogen accumulation in liver cells. In addition, as shown in Fig. 5D, obvious reticular

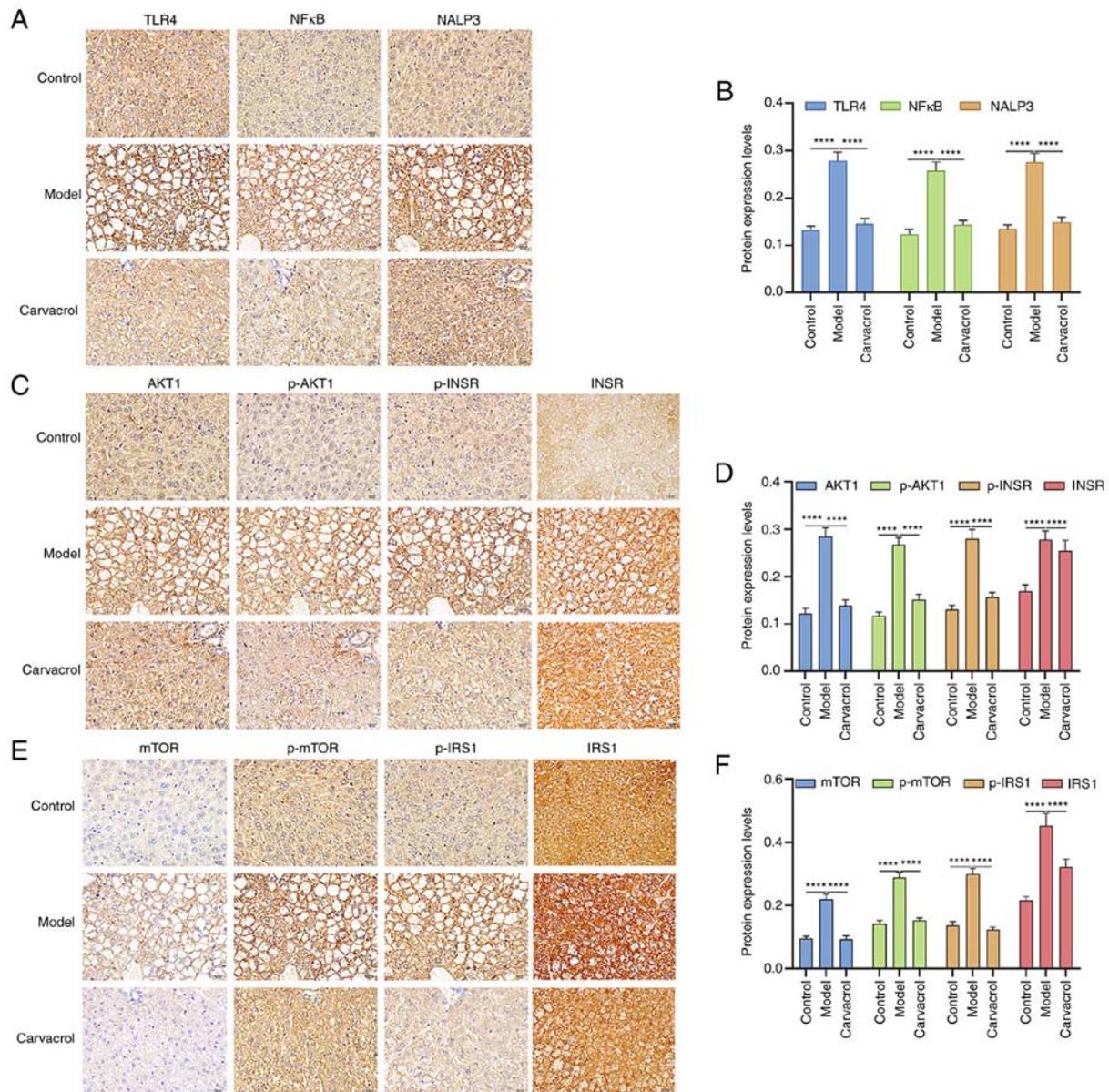


Figure 6. Immunohistochemical analysis of the expression levels of insulin, TLR4/NF- κ B and AKT1/mTOR signaling pathway components in the liver tissues of db/m+ and db/db mice. (A and B) TLR4, NF- κ B and NALP3; (C and D) AKT1, p-AKT1, INSR and p-INSR; (E and F) mTOR, p-mTOR, IRS1 and p-IRS1. Scale bar, 20 μ m; magnification, x200. ****P<0.0001. TLR4, Toll-like receptor 4; INSR, insulin receptor; IRS1, insulin receptor substrate 1; p-, phosphorylated.

fiber hyperplasia was detected in the liver tissues of db/db mice, which was notably improved by carvacrol treatment.

Effects of carvacrol on insulin, TLR4/NF- κ B and AKT1/mTOR signaling pathways in db/db mice. To assess the potential mechanisms underlying the effects of carvacrol on T2DM-induced liver injury, the expression of insulin, TLR4/NF- κ B and AKT1/mTOR signaling pathway components were detected in the liver tissue specimens. Immunohistochemistry results demonstrated that TLR4, NF- κ B, NALP3 (Fig. 6A and B), AKT1, p-AKT1, INSR, p-INSR (Fig. 6C and D), mTOR, p-mTOR, IRS1 and p-IRS1 (Fig. 6E and F) were all highly expressed in the liver tissues of db/db mice compared with those in the control group. Following treatment with carvacrol, the expression levels of these proteins were significantly suppressed in the liver tissues of db/db mice. These data indicated that both total and phosphorylated forms of AKT,

mTOR, INSR and IRS1 were activated in the liver tissues of db/db mice and were reduced after carvacrol treatment, thus suggesting that carvacrol may inhibit the expression and phosphorylation of these proteins in db/db mice. Consistently, immunofluorescence results confirmed that the expression levels of NALP3, mTOR, p-mTOR (Fig. 7A and B), TLR4, AKT1 and p-AKT1 (Fig. 7C and D) were higher in the liver tissues of db/db mice compared with those in the control group. Conversely, carvacrol administration significantly decreased the expression levels of these aforementioned proteins in db/db mice. In addition, the expression levels of these proteins were detected in the liver tissues of the three groups using western blotting (Fig. 8A). As expected, the expression levels and the phosphorylated/total protein ratios of these proteins were significantly upregulated in db/db mice compared with those in the control group (Fig. 8B-F). Nevertheless, carvacrol treatment significantly decreased the

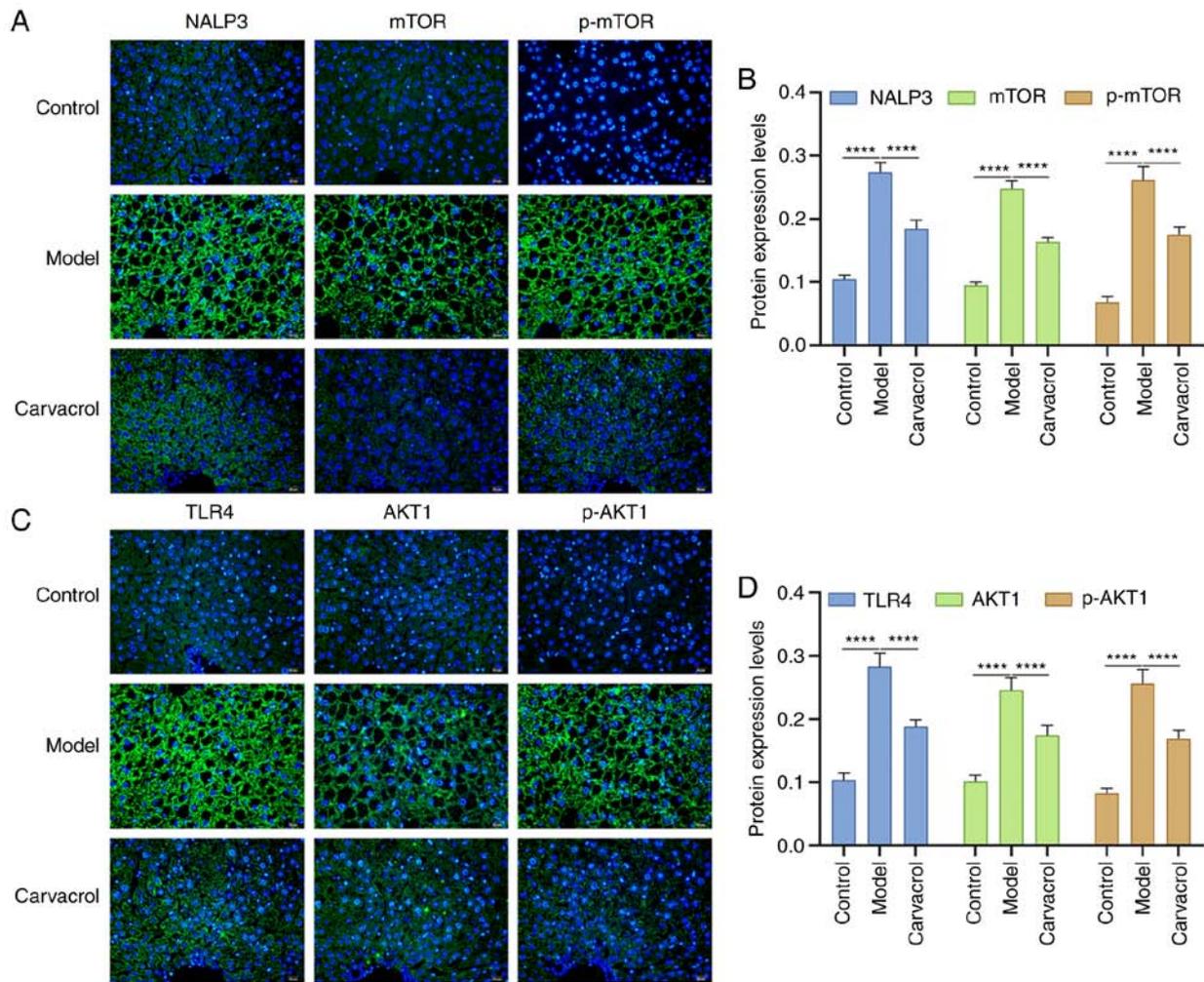


Figure 7. Immunofluorescence analysis of the expression levels of insulin, TLR4/NF- κ B and AKT1/mTOR signaling pathway components in the liver tissues of db/m+ and db/db mice. (A and B) NALP3, mTOR and p-mTOR; (C and D) TLR4, AKT1 and p-AKT1. $n=15$ /group. Scale bar, 20 μ m; magnification, $\times 200$. **** $P<0.0001$. TLR4, Toll-like receptor 4; p-, phosphorylated.

expression levels and the phosphorylated/total protein ratios of these proteins in db/db mice. These findings indicated that carvacrol could ameliorate T2DM-induced liver injury via insulin, TLR4/NF- κ B and AKT1/mTOR signaling pathways.

Discussion

NAFLD has a very high prevalence worldwide and is characterized by increased intrahepatic fat. Obesity and insulin resistance, as the two main causes of T2DM, have been shown to be significantly associated with the early stages of NAFLD, even progressing to more severe steatohepatitis and cirrhosis (24). The present study investigated the function of carvacrol in T2DM-related NAFLD treatment. The results indicated that carvacrol effectively reduced liver damage, fibrosis and liver dysfunction in db/db mice, which was associated with insulin, TLR4/NF- κ B and AKT1/mTOR signaling pathways.

In the present study, db/db mice were used as a model of T2DM. It is well known that obesity and T2DM are both risk factors of NAFLD (25). In the present study, db/db mice exhibited high body weight and liver weight, which was ameliorated following treatment with 10 mg/kg carvacrol for 6 weeks. Blood glucose is one of the most important indicators used to

observe diabetes-induced liver injury as well as the effect of drug treatment. The present study demonstrated that carvacrol administration could significantly decrease blood glucose levels in db/db mice. A previous study reported that carvacrol treatment had no effect on blood glucose and body weight for db/m+ mice compared with the controls (17), indicating that carvacrol has no toxic effect on healthy mice. Insulin resistance is a main therapeutic target for T2DM (26). In the present study, carvacrol significantly decreased serum insulin and HOMA-IR levels of model mice. Important indicators of blood lipid analysis include TC, TG, LDL-C and HDL-C (27). TG is the main energy storage unit of the human body and is an important source of energy for day-to-day activities. However, the continuous high concentration of TG in serum may lead to excessive accumulation in liver cells and can cause fatty liver disease (28). Notably, carvacrol treatment significantly lowered serum TC, TG and LDL-C levels, and increased HDL-C levels in db/db mice, suggesting that carvacrol could ameliorate dyslipidemia.

High concentrations of AST and ALT exist in liver cells; ~80% of AST is contained in the mitochondria and ALT is only distributed in the cytoplasm (29). If liver cells are seriously damaged, AST and ALT are released into the blood; therefore, serum AST and ALT levels are important indexes for liver injury.

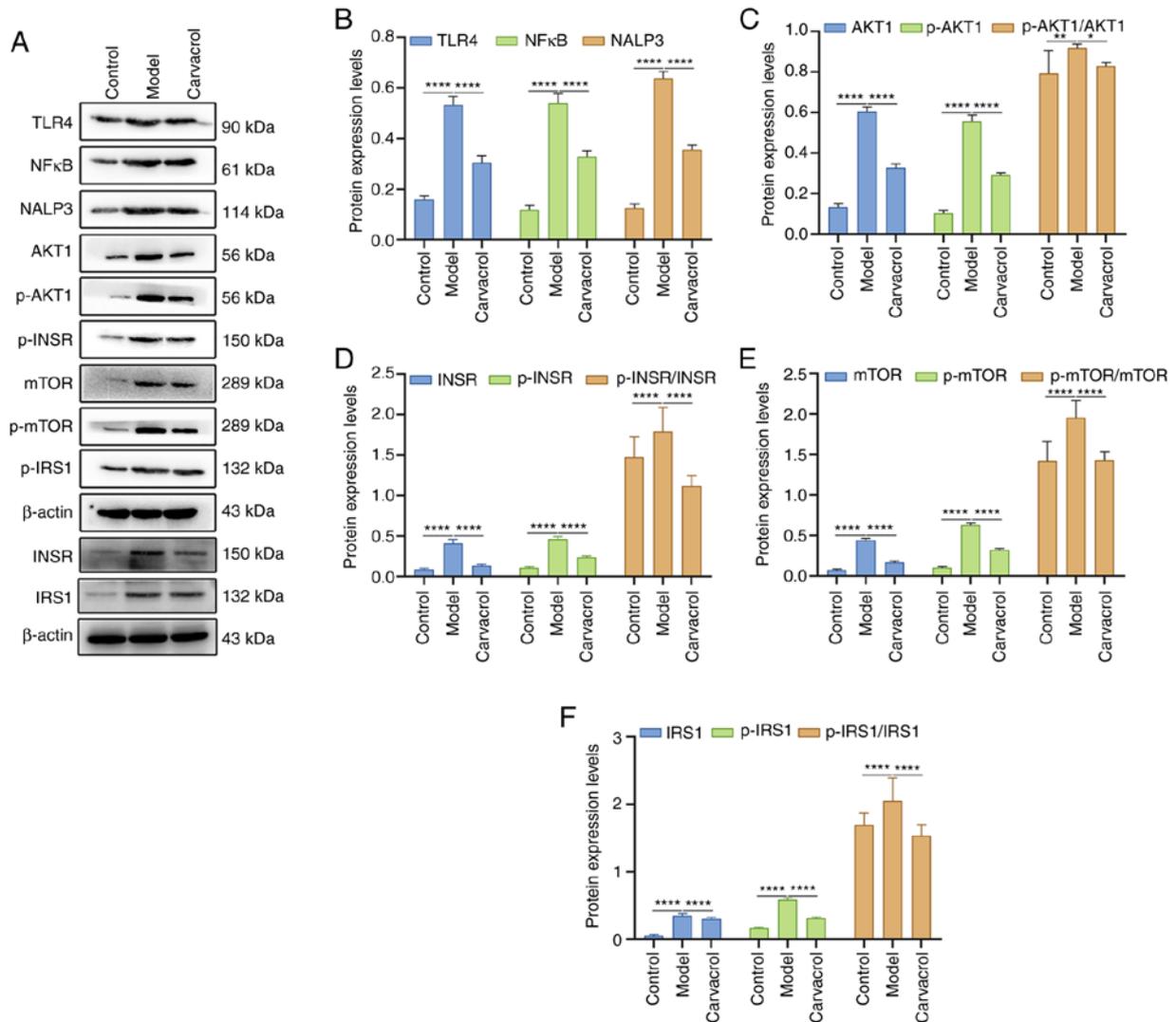


Figure 8. Western blot analysis of the expression levels of insulin, TLR4/NF- κ B and AKT1/mTOR signaling pathway components in the liver tissues of db/m+ and db/db mice. (A) Representative western blot images. Total INSR and IRS1 (and β -actin) were blotted for on a different membrane from the other proteins. (B) TLR4, NF- κ B and NALP3; (C) AKT1, p-AKT1 and p-AKT1/AKT1; (D) INSR, p-INSR and p-INSR/INSR; (E) mTOR, p-mTOR and p-mTOR/mTOR; (F) IRS1, p-IRS1 and p-IRS1/IRS1. β -actin was used as a loading control. n=15/group. *P<0.05; **P<0.01, ****P<0.0001. TLR4, Toll-like receptor 4; INSR, insulin receptor; IRS1, insulin receptor substrate 1; p-, phosphorylated.

In the present study, db/db mice exhibited high serum AST and ALT levels, suggesting liver cell damage. Conversely, carvacrol significantly ameliorated liver function in db/db mice, indicating that carvacrol may possess a certain protective effect on the liver. In a previous study, obese diabetic db/db mice manifested renal injury (30). The present findings indicated that carvacrol could improve renal function of db/db mice. Furthermore, the results of pathological analysis demonstrated that severe liver steatosis and fibrosis was present in db/db mice; however, following administration of carvacrol for 6 weeks, liver steatosis and fibrosis were markedly ameliorated. In NAFLD, steatosis has been reported to be present in >5% of liver cells in human patients (31). It has been reported that carvacrol could relieve the progress of carbon tetrachloride-induced rat liver fibrosis; this was associated with Hippo and TGF- β signaling pathways (32). Furthermore, carvacrol may exert a positive effect on proliferation and regeneration of liver cells following 24 and 48 h of partial hepatectomy (33). These findings suggested that carvacrol could relieve the T2DM-induced liver damage.

Pharmacologically targeting signal molecules is an attractive strategy against T2DM complications (24). The present study revealed that carvacrol administration could ameliorate liver injury in db/db mice, which was associated with insulin, TLR4/NF- κ B and AKT1/mTOR signaling pathways. Hyperglycemia, insulin deficiency and insulin resistance are the main features of T2DM (34). As an insulin-sensitive organ, the liver functions in maintaining a systemic glucose metabolism balance (34). The present results indicated that carvacrol could ameliorate liver insulin sensitivity in a mouse model of T2DM by mediating insulin signaling pathway components, including INSR, p-INSR, IRS1 and p-IRS1. A previous study reported that the TLR4/NF- κ B pathway may be involved in diabetic liver injury (35). TLR4 is one of the key members of the TLR family, which can activate the innate immune system (36). NF- κ B, as a dimeric transcription factor, can induce the expression of inflammation-related cytokines, such as TNF- α and IL-6 (37). The present data suggested that carvacrol could inactivate the TLR4/NF- κ B pathway and suppress

NALP3 expression in the liver tissues of db/db mice, thereby reducing the inflammatory response. The AKT1/mTOR pathway functions in mediating insulin resistance (38,39). The present study demonstrated that carvacrol could regulate insulin resistance by targeting the AKT1/mTOR pathway.

In conclusion, the present study reported that carvacrol administration could ameliorate liver injury, particularly T2DM-induced dyslipidemia and insulin resistance. Mechanistically, carvacrol could protect the liver in T2DM via insulin, TLR4/NF- κ B and AKT1/mTOR signaling pathways. Thus, carvacrol may be considered a promising treatment strategy for T2DM-associated liver injury and the therapeutic effect of carvacrol on T2DM-induced liver injury is worthy of further in-depth research.

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Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WZ conceived and designed the study. LC, HZ and CD conducted most of the experiments and data analysis, and wrote the manuscript. QH, YC, QW and SL conducted a small number of experiments and data analysis, and wrote and revised the manuscript. WZ and LC confirm the authenticity of all the raw data. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine (approval no. 2018072).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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